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Glyphosate-induced antioxidant imbalance in HaCaT: The protective effect of Vitamins C and E

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Abstract

Roundup 3 plus®, a glyphosate-based herbicide, is widely used in the ground, but its extensive use has posed a health risk in man. The aim of this study was firstly to investigate how glyphosate alone or included in Roundup 3 plus® affected the antioxidant defense system and lipid peroxidation of human cutaneous cells, and secondly, to evaluate the ameliorating effects of antioxidants, as Vitamin C (VitC) and Vitamin E (VitE), against Roundup 3 plus®-induced epidermal antioxidant impairment. Our results showed that glyphosate alone or included in Roundup 3 plus®, induced significant changes in cellular antioxidant status as a glutathione depletion, enzymatic (catalase, glutathione-peroxidase and superoxide dismutase) disorders, and increased lipid peroxidation. VitC or VitE supplementation increased superoxide dismutase, glutathione-reductase and -peroxidase activities and reduced lipid peroxidation in Roundup 3 plus®-treated keratinocytes. These in vitro data indicated that VitC and VitE might have preventive effects against deleterious cutaneous cell damage caused by Roundup 3 plus®.

Keywords: HaCaT; Glyphosate-based herbicides; Antioxidant status; Vitamin C; Vitamin E

1. Introduction

The skin is a barrier between our body and the outside world. In this case, the human body is exposed to a peroxidative environment that includes air pollutants (Podda and Fuchs, 2004), ultraviolet B solar light (Zhu and Bowden, 2004), cigarette smoking (Izzotti et al., 2004) or pesticides (Amerio et al., 2004). Glyphosate (G) is the active herbicide component of Roundup 3 plus® (R). Alone or in association, G was previously considered to be harmless in normal use or chronic exposure in previous testing approach (Williams et al., 2000). However, toxic activity of R at sublethal concentration has been recently demonstrated in fish (Szarek et al., 2000; Terech-Majewska et al., 2004) or in other aquatic organisms (Tsui and Chu, 2003). In man, Bradberry et al. (2004) reported the different damages that G-based formulations could provoke on several organs, and Marc et al. (2002, 2004) demonstrated that R affected the cell division at the level of the molecular switch of the CDK1/Cyclin B association. In a previous report (Géhin et al., 2005), we showed that G and R could be responsible for cytotoxic effects on human epidermal cells; therefore, we proposed that antioxidant compounds could be associated to herbicide formulations to decrease their deleterious effects on human skin.

Generation of reactive oxygen species (ROS) in the skin develops oxidative stress when their concentration exceeds the antioxidant defense ability of the target cell. Thus, to oppose this oxidative injury on the structure of lipids and proteins, human skin is equipped with a network of enzymatic and nonenzymatic antioxidant defense systems (Kohen, 1999). These nonenzymatic chemical compounds like tocopherols including Vitamin E (VitE), and ascorbates including Vitamin C (VitC), administered either topically or orally, exert a protective effect on skin cells. Enzymatic defense against ROS is performed by antioxidant enzymes such as catalase, superoxide dismutase (SOD), glutathione-peroxidase (GSH-Px), and glutathione-reductase (GSSG-Red); intracellular-reduced glutathione (GSH) also plays an important role. Consequently, oxidative stress can be defined as a redox imbalance between an excess of production of free radicals and a defect in antioxidant defense (Junqueira et al., 2004). In that context, ROS was

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supposed to be responsible for various cutaneous disorders and keratinocytes are certainly a good biological epidermal system to test the antioxidant defenses. In our study, we employed a model of epidermal cell line, HaCaT, firstly to assess the effects of G and R on the ability of human keratinocytes to withstand oxidative challenge and, secondly, to determine if supplementation with VitC or VitE could compensate for oxidative impairment in protecting cells from this aggression.

2. Materials and methods

2.1. Materials

Dubelcco's modified Eagle's minimum essential medium (DMEM), fetal calf serum (FCS), trypsin-EDTA (0.25%), Falcon 75 cm^2 flasks were from D. Dutscher (Brumath, France). Ascorbic acid, Pierce bicinchoninic acid protein assay kit, bovine serum albumin, cumen hydroperoxide, dimethylsulfoxide, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), *N*-(phosphonomethyl)-glycine (glyphosate), ferricytochrome *c*, hydrogen peroxide (H_2O_2) glutathione (GSH), glutathione reductase (GSSG-Red), oxidized glutathione (GSSG), *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT), reduced ß-nicotinamide adenine dinucleotide phosphate (ß-NADPH), superoxide dismutase (SOD), 5-sulfosalicylic acid, 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), trolox®, xanthine, xanthine oxidase were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Roundup 3 plus®, a glyphosate-surfactant herbicide (containing 21% (m/m) isopropylamine glyphosate salt (170 g/l), 8% (m/m) polyoxyethylene amine (POEA) and 71% water and others minor ingredients(m/m)) was a formulated commercial product from Monsanto (Paris, France). Phosphate-buffered saline (PBS) without calcium and magnesium was purchased from VWR International (Cergy-Pontoise, France).

2.2. Experimental procedure

2.2.1. Cell culture

HaCaT, an immortalized human keratinocyte line, was a generous gift from Nathalie Gault (Commissariat à l'Energie Atomique, Bruyères Le Châtel, France) (Boukamp et al., 1988; Gault et al., 2002).

Cells were cultured in DMEM medium supplemented with 10% (v/v) FCS, 5 M of HEPES, 80 mg/l of gentamicin in a humidified 5% CO_2 atmosphere at 37 °C. Human keratinocytes (3500 cells/cm^2) were grown to confluence (7×10^5–1×10^6 cells/ml) in 75 cm^2 culture flasks (D. Dutscher, Brumath, France). The medium was removed every 48 h and cells were subcultured every 7 days. The cells were used at passages 3, 4 or 5 after thawing.

2.2.2. Cell treatments

2.2.2.1. Cytotoxicity assay. HaCaT cells were seeded at a density of 6×10^4 cells per well in 100 μl culture medium containing 10% FCS on 96 multiwell culture plates and incubated overnight for adherence. The following day, the medium was removed and the cells were incubated in FCS-free medium containing concentrations of G or R (0, 10, 12.5, 15, 17.5, 20, 22 and 25 mM) and/or VitE or VitC (100 μM). Each experiment was performed twice, and each determination was performed in triplicate.

2.2.2.2. Enzyme activities assay. HaCaT cells were seeded in 75 cm^2 culture flasks at the density of 5×10^5 in 10 ml culture medium containing 10% FCS. After adherence, the cells were incubated in FCS-free medium containing increasing concentrations of G alone (10, 15 and 17.5 mM) or included in R (10, 15, and 20 mM) and/or VitE or VitC (100 μM) for 24 h.

2.2.3. Preparation of protein extract

Cells were treated with various chemicals as detailed in Section 2.2.2. After washing two times with ice-cold homogenization buffer, whole cells extract was obtained by using cell lifter. The cell suspension was sonicated during 20 s. The protein fraction was stored at −80 °C until use. The protein concentration in the homogenates was determined using the bicinchoninic acid protein assay kit, according to the manufacturer's instructions. Bovine serum albumin was used as a standard.

2.3. Biochemical analysis

2.3.1. Cytotoxicity assay

Cytotoxicity assay was determined according to the method of Mosmann (1983), and the MTT procedure was previously described (Géhin et al., 2005).

2.3.2. GSH content

GSH content was measured according to the DTNB recycling procedure described by Griffith (1980) and modified by Allen and Arthur (1987). Keratinocyte homogenates were deproteinised by addition of 5% sulfo-5-salicylic acid, supernatant was mixed (v/v) with DTNB (0.6 mM) and the absorbance was measured with a spectrophotometer at 405 nm immediately after mixing. Results were expressed as nmol/mg cell proteins.

2.3.3. GSSG-Red activity

GSSG-Red activity was assessed according to the procedure described by Carlberg and Mannervik (1985) modified by Bellomo et al. (1987), by measuring the disappearance of ß-NADPH (0.1 mM) at 340 nm, in the presence of GSSG (1 mM). Results were expressed as nmol GSSG reduced (=ß-NADPH oxidized)/min/mg cell proteins.

2.3.4. GSH-Px activity

GSH-Px activity was measured as described by Lawrence and Burk (1976) in the presence of GSH (2 mM), GSSG-Red (1 U/ml), ß-NADPH (0.2 mM) and cumen hydroperoxide (4 mM). GSH-Px activity was determined spectrophotometrically by following the oxidation of ß-NADPH at 340 nm. Results were expressed as nmol ß-NADPH oxidized/min/mg cell proteins.

2.3.5. Lipid peroxidation

The extent of spontaneously lipid peroxidation was evaluated by measuring the concentration of TBA reactive substances (TBARS) as described by Yagi (1976) and Ohkawa et al. (1979). Cell proteins were precipitated with 10% TCA. After centrifugation, supernatant was mixed with TBA reagent (0 67%) and the mixture was kept in boiling water bath for 15 min. The fluorescent reaction product was extracted with *n*-butanol and the fluorescence was measured in the organic phase using a spectrophotometer (excitation, 535 nm; emission, 565 nm). The assay procedure was calibrated using tetraethoxypropanone as an MDA source. Results were expressed as pmol TBARS/mg cell proteins.

2.3.6. SOD activity

SOD activity was analyzed according to the method of Mc Cord and Fridovich (1969) modified by Flohe and Otting (1984). The reduction rate of cytochrome *c* by superoxide radicals ($O_2^{\bullet -}$) was monitored at 550 nm utilizing xanthine–xanthine oxidase system as the source for $O_2^{\bullet -}$. SOD competing with cytochrome *c* for $O_2^{\bullet -}$ reduction and thus decreasing the reduction rate of cytochrome *c*. Fifty microliter of cell were used in the assay mixture under the following conditions: 50 mM phosphate buffer pH 7.4, 0.1 mM EDTA, 2 mM xanthine, 522 mM cytochrome *c* and xanthine oxidase at a concentration producing a rate of cytochrome *c* reduction of 0.025 absorbance unit/min, in the absence of SOD, in a total reaction volume of 3 ml. Under these conditions, one unit (U) of SOD is defined as the amount which causes 50% inhibition of the initial rate of reduction of cytochrome *c* and the results were expressed as U/mg proteins.

2.3.7. Catalase activity

Catalase activity was assessed as described by Aebi (1984) by spectrophotometric analysis (at 240 nm) of the rate of hydrogen peroxide decomposition (15 mM) in PBS at 25 °C. Enzyme activity was expressed as unit (*U*)/mg cell proteins, where *U* is the difference in absorbance per unit time.